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Geographic Distribution and Severity of Strobilurin Fungicide Resistance among *Rhizoctonia solani* on Rice in Southwestern Louisiana

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GEOGRAPHIC DISTRIBUTION AND SEVERITY OF STROBILURIN FUNGICIDE RESISTANCE AMONG *RHIZOCTONIA SOLANI* ON RICE IN SOUTHWESTERN LOUISIANA

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This thesis is dedicated to Stephen Harding, Lacy Brooks, Patricia Bollich, and Dr. Clayton Hollier. I will forever treasure these past years spent with you in “Rhizoctopia”.

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ABSTRACT

Strobilurin fungicide resistance threatens rice production in southwestern Louisiana. Strobilurins are the often relied upon method of managing sheath blight development due to the lack of strong genetic resistance to this disease in the currently available rice germplasm. However, isolates of *Rhizoctonia solani* AG 1-IA causing sheath blight were reported resistant to azoxystrobin near Mowata, LA in 2011. To determine the geographic distribution and severity of azoxystrobin resistance, 40 commercial rice fields in southwestern Louisiana were sampled for isolates of *R. solani*. Sensitivity to pyraclostrobin and trifloxystrobin was also assessed to investigate cross-resistance. A genetic test was used to confirm that all isolates belonged to AG 1-IA. One isolate from each field was tested using a traditional fungicide-amended solid medium assay measuring radial mycelial growth. A second, higher-throughput assay was developed to accommodate all 162 collected isolates. This assay used fungicide-amended liquid medium and measured light absorbance through suspended mycelium. Both assays were used to determine the EC_{50} (the effective concentration to inhibit mycelial growth halfway between an observed maximum and minimum response of individual dose-response curves) separately for the three strobilurins. Azoxystrobin resistance was found in a greater area than previously reported. Cross-resistance was only weakly supported between azoxystrobin and the other fungicides due to few significant differences in pyraclostrobin and trifloxystrobin responses across the isolate population. However, the sensitivity distributions were bimodal for both azoxystrobin and pyraclostrobin, while unimodal for trifloxystrobin. This clustering of higher-sensitivity and lower-sensitivity individuals at opposite ends of the spectrum suggests the presence or development of isolates resistant to azoxystrobin and pyraclostrobin, respectively. Fungicide resistance management will continue to be vital for rice production, and fungicide rotations utlizing trifloxystrobin may be useful against even azoxystrobin-resistant and possibly pyraclostrobin-resistant isolates of *R. solani*.

CHAPTER 1. INTRODUCTION AND REVIEW OF LITERATURE

Rice is the quintessential staple food across the globe. More than 20% of the calories eaten by humans are derived from rice (Smith 1998). Rice is also an important cultural staple and economic asset in Louisiana. In 2014, rice was the second most valuable row crop grown with a farm value of over $492 million (compared with soybeans, over $988 million) and the second most widely planted row crop in Louisiana with over 449,000 acres planted (compared with soybeans, at almost 1,3921,000 acres) (Louisiana Ag Summary, 2014 State Totals 2014).

1.1 SHEATH BLIGHT IN LOUISIANA

Sheath blight is a major, chronic, and in most years, the most yield limiting disease for rice growers in Louisiana (Louisiana Rice Production Handbook 2014). It was the most severe disease recorded in a 1984 state-wide Louisiana rice disease survey (Groth and Hollier 1985) and continues to be a serious threat today. Untreated, sheath blight has been shown to cause up to 40% yield loss. This loss can be reduced with a strobilurin fungicide application (Groth 2008) if the sheath blight is caused by *R. solani* that is strobilurin-sensitive.

1.1.1 CAUSAL ORGANISM. Sheath blight is caused by *Rhizoctonia solani* Kühn, a basidiomycete anamorph fungus (Kühn 1858). Its mycelia display characteristic right angle branching with a slight constriction near the origin of the branching hypha usually followed by septation. *Rhizoctonia solani* is multinucleate and not known to produce asexual spores. The teleomorph, *Thanatephorus cucumeris* (Frank) Donk, is known to produce basidiospores (Donk 1956; Frank 1883). Traditionally, the perfect state has been scarcely reported in rice fields (Rao 1995) and is thought to play little role in the disease cycle (Rush and Lee 1992). Recent population genetic studies found certain allele distributions that suggest sexual reproduction might be more common than originally thought (Padasht-Dehkaei et al. 2013; Rosewich et al. 1999). Basidiospore production can be induced from vegetative mycelia *in vitro* (Oniki, Ogoshi, and Araki 1986).

Rhizoctonia solani can be subdivided into anastomosis groups (AGs). Members of each AG will typically fuse (anastomose) with each other, signifying recognition of 'self 'or a near clonal isolate. Different AGs often preferentially infect different host plants, causing disparate

diseases (González García, Portal Onco, and Rubio Susan 2006). *Rhizoctonia solani* from AG-1 Intraspecific Group A (IA) causing sheath blight on rice is the specific focus of this study.

1.1.2 DISEASE CYCLE. Unlike most other AGs, sheath blight-causing *R. solani* typically infects aerial parts of the rice plant. Symptoms first appear on rice sheaths or leaves at the flood waterline where inoculum floats in the form of sclerotia and mycelia-infected plant tissue from previous crops or weeds. Hyphae then grow over the surface of the rice tissue, forming infection cushions to draw nutrients from the plant cells (Rush and Lee 1992). A toxin is released from the mycelium (Vidhyasekaran et al. 1997) that causes oblong, rounded lesions with gray-green or straw-colored centers and yellow to brown margins. Initial lesions are often elliptical, while a snakeskin pattern emerges as the disease progresses and lesions coalesce. The fungus progressively grows along the plant's surface, infecting tissues up to and including the flag leaf and panicle. The pathogen can spread to an adjacent plant through leaf-to-leaf contact. Dark sclerotia are produced on leaves and sheaths as overwintering structures, forming from ovular monilioid cells (Rush and Lee 1992).

1.1.3 MANAGEMENT. Management of *R. solani* can be difficult for several reasons. Being a soilborne organism, *R. solani* can persist in the soil as sclerotia for multiple years and is now endemic to most areas where rice is produced (Rush and Lee 1992). Crop rotation is generally not effective because of a wide host range that spans many crops and weeds. *Rhizoctonia solani* causes aerial blight on soybeans, which are often rotated with rice in southwestern Louisiana fields. It also infects other leguminous crops including mung and common bean (Rush and Lee 1992). Under disease-favoring environmental conditions, *R. solani* can also infect corn, sorghum, and sugarcane (Louisiana Rice Production Handbook 2014).

Several other modern production practices contribute to sheath blight severity. The global demand for greater crop yield has driven the development of more input intensive agricultural techniques. Fertilizers and densely grown crops have subsequently increased yields. Higher nitrogen content in rice leaves due to fertilizers can increase sheath blight severity. Dense rice stands with early-closing canopies can also contribute to greater disease

pressure by increasing microclimate humidity, as high humidity favors pathogen development (Louisiana Rice Production Handbook 2014; Rush and Lee 1992).

Another factor in disease severity is the type of rice being grown in southern Louisiana. In a controlled experiment in 2008, unsprayed, inoculated rice fields in Louisiana resulted in grain yield losses of 22-34% for cultivars susceptible or very susceptible to sheath blight, 14-16% for unsprayed moderately susceptible cultivars, and 5% for a moderately resistant cultivar (Groth 2008). Short- and medium-grain rice cultivars tend to be more resistant to sheath blight than long-grain cultivars (Louisiana Rice Production Handbook 2014; Rush and Lee 1992), but long-grain cultivars dominate Louisiana production. Only 5.8, 9.5, 5.5, and 4.3% of rice acreage during 2010, 2011, 2012, and 2013, respectively, produced medium-grain rice; the remaining rice acreage was dedicated to long-grain rice cultivars (Salassi, Webster, and Wilson 2014; Salassi, Wilson, and Walker 2012). Fortunately, several modern long-grain cultivars, such as Arkansas' 'Taggart' and 'Roy J' and RiceTec's 'CLXL745' and 'XL753', have an improved sheath blight resistance rating of moderately resistant instead of the susceptible or very susceptible ratings typical of long grain rice (Saichuk et al. 2014). Taggart and Roy J have not seen much use in Louisiana, but CLXL745 has been planted on about 11, 15, 10, and 14% of all Louisiana rice production acreage during 2010-2013, respectively (Salassi, Webster, and Wilson 2014; Salassi, Wilson, and Walker 2012). Currently, there are no highly resistant rice cultivars available, as the greatest available sheath blight resistance is only moderately resistant.

There have been meager successes using biocontrol methods to manage sheath blight. Two prominent examples are commercial formulations of *Bacillus subtilis*: Integral™ (BASF) and Serenade Max™ (Bayer CropScience). Integral™ was applied three times to rice plants and resulted in higher grain yields than a single-application fungicide control under transplanted field conditions (Vijay Krishna Kumar et al. 2012). Serenade Max™ applied at the boot stage consistently protected rice yield, though no fungicide control was compared (Zhou and McClung 2013). Non-commercial biocontrol formulations are less supported by research. Culture filtrates of *Chaetomium aureum* reduced sheath blight disease as much as a fungicide application, but yield effects were not assessed (Wang et al. 2013). Three foliar applications of

Desmos chinensis plant extract were shown to reduce sheath blight severity in transplanted pot conditions, while three applications of fungicide reduced severity further (Plodpai et al. 2013). Three formulations of the chemical chitosan were shown to reduce sheath blight incidence of potted rice plants, but comparison to a fungicide treatment was not provided (Liu et al. 2012). Investigations have been done on other *R. solani*-plant interactions including the use of compost, other organic amendments, and *Streptomyces* spp. (Blok et al. 2000; Kanini et al. 2013; Tuitert, Szczech, and Bollen 1998). One final potential mode of biocontrol is associated with "Rhizoctonia decline", a disease that shrivels mycelia of *R. solani* and severely reduces pathogenicity on plant hosts. However, this method has limited efficacy unless a near-clonal *R. solani* isolate infected with Rhizoctonia decline can be located, maintained, and deployed in each field (Castanho and Butler 1978a, b; Castanho, Butler, and Shepherd 1978; Lakshman, Jian, and Tavantzis 1998).

Often the most effective and recommended management of this disease is application of strobilurin fungicides. When appropriately timed around the boot stage of rice development to 50% heading (Groth 2005; Saichuk et al. 2014), a strobilurin fungicide application can reduce *strobilurin-sensitive* sheath blight's impact on a crop. Yield losses of 8% to 40% were demonstrated when comparing inoculated field plots with an application of azoxystrobin or no application across six rice cultivars ranging from very susceptible to moderately resistant to sheath blight (Groth 2008).

1.2 STROBILURINS AND FUNGICIDE RESISTANCE

Strobilurins are a class of fungicides first released for sale in 1996, the year that Syngenta and BASF released azoxystrobin and kresoxim-methyl, respectively (Bartlett et al. 2002). Strobilurins affect plant pathogenic fungi by inhibiting cellular respiration (Bartlett et al. 2002; Becker et al. 1981). Fungicide molecules occupy the Qo (quinol oxidation) site on the mitochondrial membrane's cytochrome bc1 complex. This occupation blocks electron flow and, consequently, respiration. This inhibition starves fungal cells of adenosine triphosphate (ATP), thus slowing their cellular activity and growth. Due to blocking the Qo site, strobilurins are considered QoI's (Qo Inhibitors), which are classified as Fungicide Resistance Action

Committee (FRAC) code C3 fungicides (FRAC 2015). Strobilurins have useful activity against many pathogens from diverse fungal groups, including oomycetes, basidiomycetes, and ascomycetes, giving them the greatest activity spectrum of any agricultural fungicide (Carlile 2012). Strobilurins are used on monocot, dicot, agricultural, and horticultural crops. In 1999, azoxystrobin had the distinction of being "the world's biggest selling fungicide", having labels for 84 different crops in 72 countries (Bartlett et al. 2002). In 1998, however, *Blumeria graminis* f. sp. *tritici* samples resistant to trifloxystrobin were collected from northern Germany, marking the first recorded incidence of a plant pathogenic fungus displaying resistance to a strobilurin fungicide (Sierotzki, Wullschleger, and Gisi 2000).

1.2.1 CYTOCHROME B MUTATIONS. A cytochrome b mutation was identified in the fungal mitochondrial DNA (mtDNA) from samples of the first strobilurin-resistant plant pathogen (*Blumeria graminis* f. sp. *tritici*), indicating that this mutation strongly influences resistance. The 143rd amino acid in the cytochrome b protein had been changed into an alanine (A) instead of a glycine (G) (Sierotzki, Wullschleger, and Gisi 2000). This point mutation is known as G143A, and it is one of several known mitochondrial mutations that can alter strobilurin sensitivity by changing the active site. Almost all field resistant plant pathogenic fungi are reported with having a cytochrome b mutation. FRAC (FRAC QoI Working Group 2006) and Ishii (Ishii 2010) agreed that three prominent cytochrome b point mutations confer QoI resistance: G143A, F129L, and G137R. As of 2012, the G143A mutation is most often reported according to FRAC (30 out of 40 plant pathogenic fungal species on FRAC's species list), which confers a strong resistance, typically requiring more than 100 times the fungicide concentration to provide equivalent control *in vitro* (FRAC QoI Working Group 2012). Fewer pathogens have the F129L mutation (8 out of 40 listed), which accompanies a typically weaker (partial) resistance, usually requiring less than 50 times the fungicide concentration. Even fewer pathogens possess the G137R mutation (1 out of 40 listed), which forms a partial resistance similar to that of the F129L mutation.

There are a number of other known cytochrome b mutations that have been identified in conjunction with reduced QoI sensitivity in organisms beyond plant pathogens. Molecular

studies regarding the natural resistance of strobilurin-producing basidiomycete fungi (Kraiczy et al. 1996) and other organisms, including ascomycete yeasts (Di Rago, Coppee, and Colson 1989), showed differences in the cytochrome b genes, each changing one amino acid in the cytochrome b protein. In 1996, a collection of these known cytochrome b point mutations and their effects was published (Brasseur, Saribaş, and Daldal 1996). This collection includes 77 point mutations in the prokaryotic cytochrome b as well as the corresponding eukaryotic mutations, highlighting the vast amount of work being done on cytochrome b and the bc1 complex at the time. Generally, mutations beyond G143A, F129L, and G137R have not been shown to reduce plant pathogenic fungi's field sensitivity to QoI's.

Identification of a cytochrome b mutation can help explain fungal insensitivity to strobilurin and QoI fungicides, but the simple presence or absence of mitochondrial mutation is not the only important consideration. Certain structures of the cytochrome b gene could prevent the G143A mutation in an organism, but some isolates of a fungal species may or may not have this nucleic acid structure. Different amounts of mutant mitochondrial DNA due to heteroplasmy can affect the fungal sensitivity to QoI's. Further, some QoI-resistant organisms do not possess a mitochondrial mutation at all.

Though the G143A mutation generates strong resistance, this mutation can be precluded by an intron located between codons 143 and 144 of the cytochrome b gene (FRAC QoI Working Group 2011). An investigation by Grasso, *et al*. (2006) sequenced 23 plant pathogen cytochrome b genes including basidiomycetes, ascomycetes, and an oomycete. The study found that the G143A mutation was never included in an organism that also possessed an intron directly after codon 143. The codon change from glycine to alanine in the presence of the intron is thought to interfere with pre-mRNA splicing, which would result in a fatal lack of cytochrome b (Grasso et al. 2006). Further studies have supported this pattern, such as one examining *Monilinia fructicola*, the causal agent of stone fruit brown rot (Luo et al. 2010), and *Podosphaera leucotricha*, which causes apple downy mildew (Koch, Felsenstein, and Stammler 2015). While this intron may prevent the G143A mutation in a fungal isolate, all isolates within a species may not share the same intron pattern. For example, isolates of gray mold (*Botrytis*

cinerea) were found to have either three or four introns in the cytochrome b gene: the former being susceptible to the G143A mutation and the latter possessing the intron that restricts G143A (Banno et al. 2009). *Pestalotiopsis longiseta*, grey blight on tea, also has multiple different intron structures within the cytochrome b gene, though not at the 143 position (Yamada and Sonoda 2012). Thus, detection of an intron at position 143 can be used to discuss the susceptibility of the specific isolate identified, but caution must be exercised when extrapolating to the entire pathogen species population.

Mitochondrial heteroplasmy as well as mechanisms outside of mtDNA have been investigated as sources of differing QoI sensitivity within a single pathogen species. Mitochondrial heteroplasmy can produce varying levels of fungicide sensitivity. For example, in grapevine powdery mildew samples, the proportion of azoxystrobin-resistant oospores correlated with the proportion of mutant mitochondrial alleles compared with the wild-type (Toffolatti et al. 2007). Similarly, samples of the pistachio Alternaria late blight pathogen displayed high levels of the mutant cytochrome b allele (greater than 70% and often greater than 90%) in azoxystrobin-resistant isolates (Ma and Michailides 2004). This suggests that typically not all of the mitochondria within a fungal isolate share the same cytochrome b mutation, or lack thereof, even in fungicide-resistant isolates. Thus, among multiple caveats, nucleic acid molecular methods must be interpreted with caution and should not be the sole method used to screen for QoI resistance in plant pathogens.

1.2.2 OTHER MODES OF RESISTANCE. It must be noted that not all field-resistant pathogens can be linked to a mitochondrial mutation. Some strobilurin-resistant isolates of cucurbit powdery mildew (*Podosphaera fusca*) and apple scab (*Venturia inaequalis*) have been found to lack cytochrome b mutations (Fernández-Ortuño et al. 2008; Köller et al. 2004; Steinfeld et al. 2001) while other resistant isolates possess the mutations (Ishii et al. 2001; Köller et al. 2004; Zheng, Olaya, and Köller 2000). Two suggested causes of the QoI resistance are a possible alteration of the Reiske iron-sulfur protein (ISP) that also forms part of the Qo pocket (Fernández-Ortuño et al. 2008) and an as of yet unknown respiratory pathway (Steinfeld et al. 2001), but neither hypothesis has been substantiated.

Alternative oxidase (AOX) can be involved in QoI resistance in plant pathogens. AOX provides an alternative route for electrons when the main mitochondrial electron transport chain is broken, whether by a QoI (mitochondrial complex III inhibitor) or another respiratory inhibitor, such as cyanide (complex IV inhibitor) or rotenone (complex I inhibitor). Oxidative phosphorylation through AOX can only produce a fraction of the cellular energy generated through the cyanide-sensitive pathway given the same electron flow (Siedow and Berthold 1986). Despite this, AOX activity has been shown to increase the amount of fungicide necessary to suppress several pathogens *in vitro*. *Pyricularia grisea* (gray leaf spot on turf) and *Venturia inaequalis* require less fungicide to suppress growth when treated with salicylhydroxamic acid (SHAM, an AOX inhibitor) (Kim et al. 2003; Steinfeld et al. 2001). *Pyricularia grisea* appears to be less sensitive to fungicide and SHAM combinations during mycelial growth, as compared to conidial germination, while *V. inaequalis* mycelia are more sensitive than conidia.

Certain efflux transporters in *Mycosphaerella graminicola* (septoria leaf blotch) have been shown to increase resistance to trifloxystrobin in a wild-type cytochrome b strain, but resistance based on efflux transporters alone seems to be of lesser magnitude than resistance based on the G143A cytochrome b mutation (Roohparvar et al. 2008). Transporters in *Pyrenophora tritici-repentis* (wheat tan spot) have been shown to allow disease development on wheat leaves treated with strobilurin fungicide, but it is unknown if the isolates tested have any cytochrome b mutations (Reimann and Deising 2005).

1.3 STROBILURIN FUNGICIDES FOR *RHIZOCTONIA SOLANI* IN LOUISIANA

Rhizoctoni solani with low response to azoxystrobin fungicide treatments was first reported in 2011 from rice plants exhibiting sheath blight in Acadia Parish, Louisiana (Olaya et al. 2012). A follow-up study in 2012 found the strobilurin-insensitive fungus only in fields within 40 km of the original location (Olaya et al. 2013). The F129L mutation was present in these Louisiana isolates, suggesting this mutation led to the decreased strobilurin sensitivity. Strobilurin resistance was also found in *R. solani* AG 1-IA isolates collected from Arkansas (Castroagudin et al. 2013) and in AG 3 isolates from Tunisia (Djébali et al. 2014). The present

study will investigate the current strobilurin insensitivity situation in southwestern Louisiana rice.

At the epicenter where strobilurin-resistant *R. solani* was first reported in Louisiana, strobilurins no longer reduce sheath blight damage to acceptable levels. While only the F129L cytochrome b mutation has been identified in *R. solani*, development of other cytochrome b mutations is possible, potentially worsening the problem. A limited number of *R. solani* entries in public gene databases provide information about this fungus' cytochrome b gene structure. GenBank has one *R. solani* entry, a specimen from AG 3. Its cytochrome b gene appears to possess six exons and five introns- one intron after the 68th, after the 132nd, within the 166th, after the 273rd, and within the 290th codon (GenBank Gene ID 16029565). If all *R. solani* isolates follow this intron structure, development of G143A causing strong strobilurin resistance is possible due to lacking an intron following codon 143.

Other fungicides are labeled for use on rice in Louisiana. At the advent of azoxystrobin resistance in Louisiana, there were propiconazole, flutolanil, and strobilurin chemistries available. Propiconazole had the least activity; flutolanil, trifloxystrobin, trifloxystrobin/propiconazole and propiconazole/azoxystrobin mixtures had intermediate activity; and azoxystrobin alone had the best activity on sheath blight, barring fungicide resistant isolates (Groth and Hollier 2014). Fluoxastrobin is also labeled for aerial blight management on soybeans. As azoxystrobin efficacy deteriorated in certain production areas, a FIRFA Section 18 request was filed in early 2012 for emergency chemical relief from sheath blight damage (Rossi 2012a). Fluxapyroxad use was then permitted temporarily for sheath blight management on rice in 2012 and 2013 (Rossi 2012b, 2014) as a result of Section 18 petitioning and was subsequently granted a full label in 2014 (Environmental Protection Agency 2014a, 2014b).

This survey will illustrate the geographic reach of *R. solani* resistant to strobilurin fungicides. Development of resistance to sheath blight has not been highly successful in rice cultivars, and rice production currently relies on fungicide use to manage sheath blight. Management techniques will need to be implemented to combat sheath blight's impact on rice

crops in southwest Louisiana. The geographical extent of resistance will highlight the importance of minimizing the spread of contaminated materials and the need for resistance management. For example, farm equipment will need to be cleaned after use in fields known to harbor resistant sheath blight to remove pathogen propagules before use in other fields. Use of strobilurins in fields with known resistance must also be carefully managed to reduce further resistance development. This can also reduce the use of fungicide that is no longer effective. This research will increase the knowledge base necessary to help prolong the life of strobilurin fungicides and begin the quest for other long-term solutions for managing this important rice disease.

1.4 PROJECT OBJECTIVES

This Master's project will study *Rhizoctonia solani* causing sheath blight disease on rice crops in southwest Louisiana and investigate the current situation of strobilurin insensitivity. Pathogenic fungal isolates will be collected, stored, and tested for fungicide resistance to strobilurins. The first major objective is to collect and preserve *R. solani* AG 1-IA samples from commercial fields. Second, isolates will be characterized by strobilurin sensitivity to develop a geographic distribution of fungicide resistance. The third objective is to determine if known cytochrome b point mutations correlate with strobilurin sensitivity levels.

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CHAPTER 2. AMENDED MEDIA FUNGICIDE SENSITIVITY ASSAYS

2.1 INTRODUCTION

The basidiomycete anamorph fungus *Rhizoctonia solani* Kühn, belonging to anastomosis group (AG) 1-IA, causes sheath blight of rice (Kühn 1858; Yang and Li 2012). The teleomorph, *Thanatephorus cucumeris* (Frank) Donk, is known but thought to be relatively unimportant in the disease cycle (Dath 1990; Donk 1956; Frank 1883). Inoculum can readily overwinter in the form of sclerotia and infected plant tissue, and the fungus is known to infect many weed and crop plant species. Inoculum can persist across multiple growing seasons and is naturally found virtually wherever rice is grown, making sheath blight one of the most severe diseases for rice growers around the world (Dath 1990; Rao 1995; Rush and Lee 1992).

Rice in an economically important crop in Louisiana, with over 181,000 hectares planted in 2014 (Louisiana ag summary, 2014 state totals 2014). It is often rotated with soybeans, on which *R. solani* causes aerial web blight (Louisiana Rice Production Handbook 2014). Sheath blight is frequently the most severe disease affecting rice in Louisiana, and yield losses of 14-34% have been demonstrated on moderately to very susceptible cultivars (Groth 2008; Groth and Hollier 1986; Rush and Lee 1992). During the 2010 through 2013 growing seasons, 74-80% of the rice acreage in Louisiana was planted with cultivars rated as very susceptible, susceptible, or moderately susceptible to sheath blight (Salassi, Webster, and Wilson 2014; Salassi, Wilson, and Walker 2012). While there are some moderately resistant cultivars available, such as RiceTec's CLXL745, they are not widely planted. There are no cultivars rated as resistant or very resistant to sheath blight presently available (Saichuk et al. 2014).

Sheath blight management then relies on fungicide treatment. The best fungicide for sheath blight management was found to be azoxystrobin alone (Quadris™) when comparing fungicides with active ingredients of propiconazole, trifloxystrobin, azoxystrobin, flutolanil, or propiconazole mixed with either azoxystrobin or trifloxystrobin (Groth and Hollier 2014). An application of azoxystrobin can prevent demonstrated yield losses of 8 to 40% when applied during the boot to 50% heading growth stages (Groth 2005, 2008). However, azoxystrobin

protection has been challenged by the 2011 discovery of *R. solani* exhibiting low fungicide response in Acadia Parish, Louisiana. Resistant isolates were then shown in 2012 to exist only within 40 km of the original detection field (Olaya et al. 2012, 2013). Strobilurin-resistant sheath blight isolates were also reported in Arkansas (Castroagudin et al. 2013).

A new fungicide active ingredient, fluxapyroxad, was given an emergency label for Louisiana in response to the azoxystrobin resistance in *R. solani* isolates and has since received a full use label. This is an effective tool for sheath blight management. However, fluxapyroxad may not provide the same protection from a number of diseases that strobilurin fungicides can manage. Specifically, trifloxystrobin (and azoxystrobin to a slightly lesser extent) has excellent efficacy on rice blast, another major rice disease, while fluxapyroxad has little to no effect. Cross-resistance among the azoxystrobin and trifloxystrobin was investigated for this reason. Pyraclostrobin was also included because it can be used to manage aerial web blight on soybeans.

Cytochrome b is the fungal protein targeted by strobilurin fungicides. Also known as QoI's, or quinol outside inhibitors, these fungicides act at and occupy the mitochondrial Qo site of cytochrome b, impairing fungal respiration by preventing electron transport (Becker et al. 1981). Cytochrome b mutations have been shown to contribute field resistance to strobilurin fungicides in plant pathogens and are thought to alter the shape of the Qo active site (FRAC QoI Working Group 2006; Ishii 2010). Only the F129L, G137R, and G143A mutations have been shown to cause field-level resistance. The resistance with F129L and G137R mutations is considered less severe than with the G143A mutation. The azoxystrobin-resistant isolates of *R. solani* from Louisiana were found to have the F129L cytochrome b mutation (Olaya et al. 2012).

Alternative oxidase (AOX) activity can also be related to strobilurin resistance. Some plants and fungi express the AOX mitochondrial membrane protein, which provides an alternate route for mitochondrial electron transport that bypasses cytochrome b (Henry and Nyns 1975). This potential avoidance of cytochrome b blockage could contribute to mitigation of strobilurin efficacy in plant pathogens (Affourtit, Heaney, and Moore 2000; Xu et al. 2013). The AOX pathway is often thought to be suppressed *in planta* by the plant's flavone

production yet active *in vitro*, confounding strobilurin sensitivity measurements (Mizutani et al. 1996; Wood and Hollomon 2003). Inhibitors of AOX, such as salyicylhydroxamic acid (SHAM) and propyl gallate (PG), are thus often added to fungicide-amended media to prevent strobilurin circumnavigation *in vitro* (Parrish and Leopold 1978; Schonbaum et al. 1971). One report suggests that while *R. solani* mycelia can be highly sensitive to azoxystrobin, this inhibition is not highly sensitive to SHAM, even though AOX appears to be expressed constitutively (Jin et al. 2009).

This study explored both AOX inhibitor and strobilurin effects on mycelial growth of *R. solani* collected from southwestern Louisiana rice fields. An existing DNA test was adapted for isolate verification and compared against traditional methods. Inhibitors of AOX were evaluated for potential use in fungicide sensitivity assays. Sensitivity to strobilurins azoxystrobin, pyraclostrobin, and trifloxystrobin was examined for isolates from geographically diverse fields to guide future sheath blight management decisions. Conventional solid media fungicide sensitivity assays were conducted on a subset of isolates, and a liquid medium assay was developed to test more isolates with the goal of reducing materials, labor, and time.

2.2 MATERIALS AND METHODS

2.2.1 ISOLATE COLLECTION AND STORAGE.

Commercial rice production fields spanning southwestern Louisiana were sampled based on a 5-mile grid. Portions of Acadia, Evangeline, Jefferson Davis, and Vermillion parishes were extensively sampled with 11, 9, 8, and 6 sites respectively; one site from each of Allen, Calcasieu, Cameron, and St. Landry parishes were also sampled, composing a total of 38 sites (Figure 2.1). Samples were also included from a known resistant field. One particularly insensitive isolate was used as a positive (resistant) control. The samples described were collected in the summers of 2013 and 2014. An isolate collected from the LSU AgCenter H. Rouse Caffey Rice Research Station in 1972, before the introduction of strobilurins, served as a negative (sensitive) control. It has been maintained on rice nodes and is routinely used to screen rice germplasm for sheath blight resistance, confirming the isolate's continued virulence.

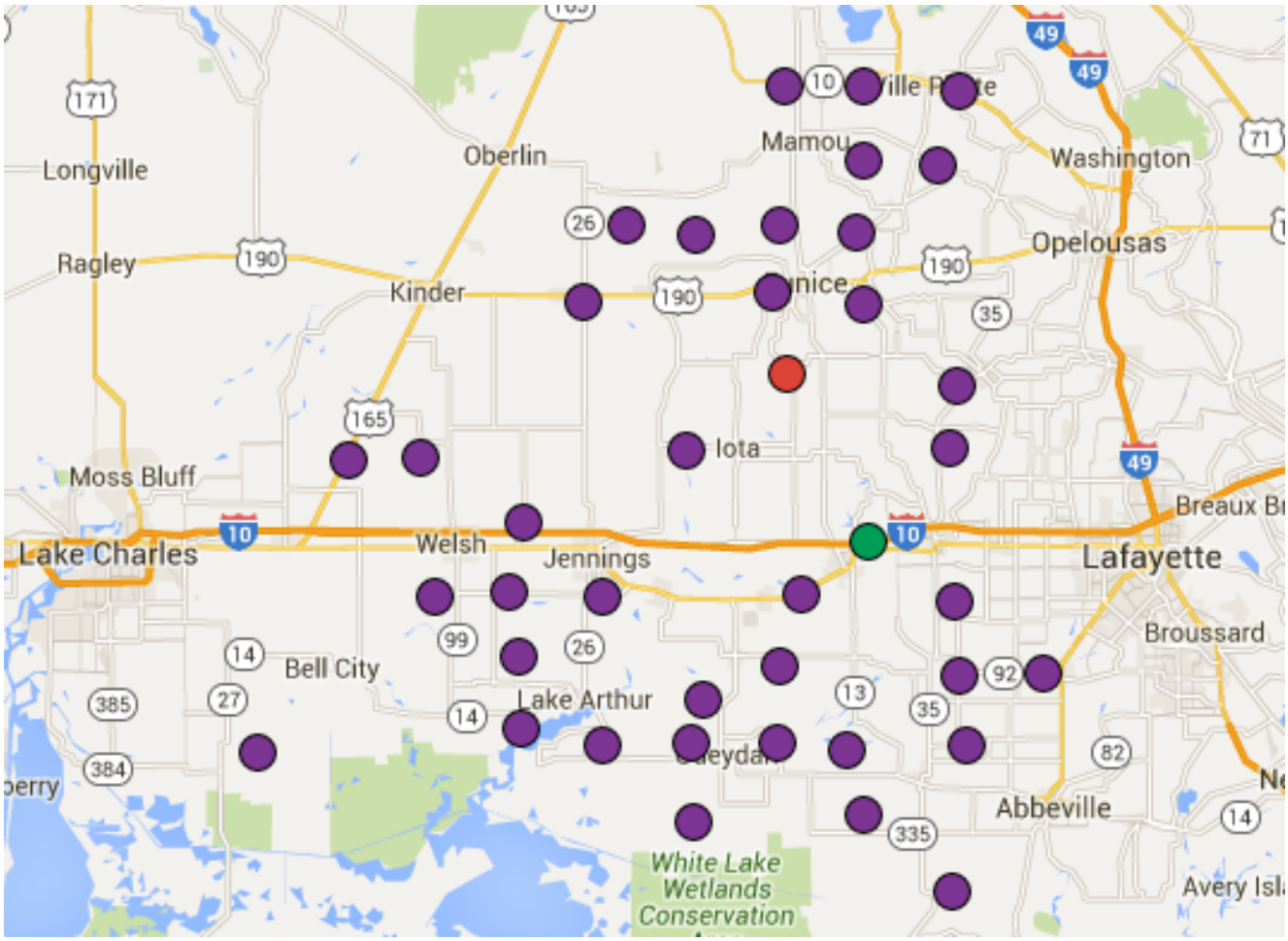

Figure 2.1. Isolate sampling sites. Purple dots represent rice fields sampled based on a 5-mile grid. The red dot marks the location of the resistant control field, and the green dot marks the sensitive control field.

Upon arriving at a selected field, the GPS coordinates were recorded. Two collectors entered from different points on the edge of the field and proceeded on non-intersecting paths until symptomatic tissue could be located. Symptomatic leaf and sheath tissue containing both a sheath blight lesion and surrounding healthy tissue were hand collected and placed in 1 quart volume plastic zip-closure bags. Multiple tissue samples were taken if possible, from the initial and adjacent plants, at each stop and pooled into one sample bag. Ten paces were required before stopping again to collect a new sample. No more than 10 samples were collected from one field, but in extreme cases as few as one sample was located. Sample bags were chilled on ice in a cooler for transport.

For isolation, lesions were scanned under a stereomicroscope. If large, characteristic right-angle mycelia were located, the lesion was excised and soaked in a 10% bleach solution for 30 seconds. The tissue was then submerged in three successive sterilized distilled water baths and dried on sterile filter paper. The dried lesion was sectioned, placed on the surface of 2% water agar, and incubated at room temperature. If no mycelia were found on a sample, symptomatic tissue was incubated in a small, moist chamber for up to several days and inspected for mycelial growth. Water agar cultures were scrutinized daily for characteristic mycelia, and selected hyphal tips were transferred to full strength potato dextrose agar (PDA, 39 g powder liter^{-1}). If bacterial contamination was present, hyphal tips were transferred to 1.8% water agar media containing streptomycin (as streptomycin sulfate at 100 mg liter^{-1}) and penicillin (as penicillin-G sodium salt at 100 mg liter^{-1}) (Gutierrez, Shew, and Melton 1997) before being transferred again to PDA. All 162 isolates were tested for the presence of bacterial contamination before storage by placing three 8.5 mm diameter agar plugs into 5 ml of nutrient broth and incubating for at least 18 hours at 28°C.

Isolates were stored as sclerotia, mycelia-colonized filter paper circles, and on rye seeds. Mature, dark sclerotia were harvested from colonies, dried with sterile filter paper, and stored in 2 ml hermetically-sealed vials placed inside a room temperature desiccator. Filter paper circles (15-mm-diameter) were autoclave sterilized and placed flat on the surface of PDA plates. An agar plug was placed in the center of the PDA plate, and mycelia were allowed to grow across the circles. Once sclerotia matured on the plate, the circles were dried in empty petri dishes in a room temperature desiccator before being stored inside sterile aluminum foil envelopes in the same desiccator. To infest rye seed, agar squares from the actively growing colony margins were incubated with approximately 15 ml of sterile winter rye seeds (which had been soaked overnight in distilled water and autoclaved with 90 minute sterile time on two successive days) in a narrow-mouthed flask and agitated daily for 5 days. The mycelia-infested seeds were then spread on open Petri dishes and allowed to dry in a laminar flow hood for 5 days before being frozen in 15 ml centrifuge tubes at -20°C (M. L. Lewis Ivey, personal communication).

2.2.2 ISOLATE VERIFICATION. A polymerase chain reaction (PCR) assay was utilized to verify each isolate as *R. solani* belonging to anastomosis group 1-IA (sheath blight). Aerial mycelia collected with sterile toothpicks were used to extract DNA from each isolate using a modified version (S. Albu, personal communication) of the Promega Wizard Purification Kit for Cultures and Mushrooms (Promega Corporation, Madison, WI). Briefly, aerial mycelia were placed in 1.5 ml sterile microcentrifuge tubes with 300 µl of Nuclei Lysis Solution (Promega) and macerated with plastic pestles. Once ground, an additional 300 µl of Nuclei Lysis Solution was added to each tube. Tubes were incubated in a 65°C heat block for 60 minutes, agitated every 15 min, then centrifuged at 1300 rpm for 10 min. About 500 µl of supernatant was transferred to a new tube, and 200 µl of Protein Precipitation Solution (Promega) was subsequently added. Tubes were agitated for 30 s and incubated at 4°C for 2 min. After centrifuging at 1300 rpm for 10 min, approximately 600 µl of supernatant was transferred to a final tube, and 600 µl of 100% isopropanol was added. Tubes were gently agitated and centrifuged at 1300 rpm for 10 min again. Liquid was decanted and pellets were washed by adding 600 µl of 70% ethanol, gently agitating, and centrifuging a final time at 1300 rpm for 10 min. Liquid was again decanted, and tubes were inverted and left to dry in a dark drawer for 60 min before DNA pellets were dissolved in 30 µl of $T_{10}E_1$ buffer (10 mM tris-HCl and 1 mM ethylenediaminetetraacetic acid). As needed, tubes were gently heated in a 50°C heat block with agitation at 15 min intervals to aid in dissolving DNA pellets.

Extracted DNA was diluted to 30 ng ml^{-1} with sterile water and tested using primers Rs1F and Rs2R developed by Sayler and Yang to specifically amplify an ITS fragment found only in *R. solani* 1-IA group members (Sayler and Yang 2007). For each isolate tested, the PCR cocktail consisted of 8.5 µl sterile water, 1.5 µl of both the forward and reverse primers (10 µM each), 12.5 µl GoTaqColorless Master Mix (Promega), and 1 µl of template DNA (30 ng/µl). Thermocycler conditions were an initial melting phase at 95°C for 4 minutes, 35 cycles of 94°C melting phase for 45 seconds, 50°C annealing for 45 seconds, and 72°C extension for 45 seconds. A final extension period at 72°C was used for 7 minutes before holding the tubes at 4°C. PCR products were visualized under ultraviolet light on a 1.5% agarose gel run at 112

volts for 25 minutes. Presence of a band approximately 140 bp in size was considered a positive result. To further verify the identification, one isolate from each collection site was sent to Dr. Craig Rothrock and David Winters, Department of Plant Pathology, University of Arkansas for traditional anastomosis identification using known tester isolates.

2.2.3 ALTERNATIVE OXIDASE INHIBITOR TOXICITY ASSAYS. One isolate from each of five collection sites (including azoxystrobin-resistant and strobilurin-sensitive control isolates) was selected to assess the effect of AOX inhibitors PG and SHAM on *R. solani* mycelial growth. For both inhibitor assays, PDA was autoclaved and cooled for handling. Propyl gallate was dissolved directly into molten PDA, whereas serial dilutions of SHAM were made in methanol and added to molten PDA, with an equal amount of methanol added to the unamended SHAM control plates. Final agar media concentrations of both SHAM and PG were 0, 50, 100, and 150 μg ml^{-1}. An agar plug, 5.4 mm in diameter, taken from the growing margin of an isolate was then transferred, mycelia side down, to each of the unamended and amended PDA plates and photographed after 30 hours of incubation at 25°C in the dark. Three replicate plates were used for each isolate-chemical concentration. Assess software (ver. 2.0; American Phytopathological Society) was used to measure colony diameter digitally (Figure 2.2). The average of two perpendicular measurements per plate was used in subsequent analyses. The entire experiment was completed twice.

Analysis of variance (ANOVA) was conducted on the colony diameters using the MIXED procedure in SAS software (ver. 9.4, SAS Institute, Inc.) to determine if each chemical significantly inhibited colony growth and to determine if there were differential responses among the five isolates tested. Fixed effects of isolate, concentration, and the interaction were analyzed separately for both chemicals, and experiment was considered a block variable. Significance was evaluated at α=0.05 using Dunnett's adjustment for colony growth inhibition and Tukey's adjustment for isolate differences. The assumption of residual normality was analyzed using the Shapiro-Wilk test in SAS's PROC UNIVARIATE. Homoscedasticity was investigated using the 'group=' option in the random statement of PROC MIXED. The 'group=' option, or none at all, that produced the lowest AIC value was used.

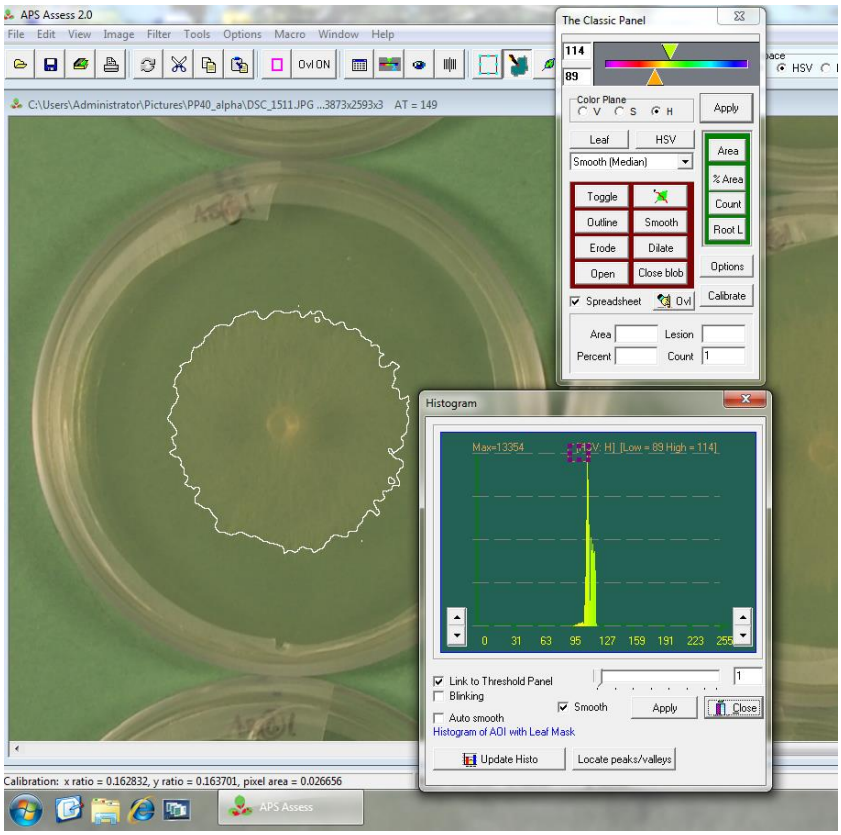

Figure 2.2. Assess 2.0 (American Phytopathological Society) software interface showing the outline of a fungal colony measured digitally.

2.2.4 SOLID MEDIUM FUNGICIDE SENSITIVITY ASSAYS. An AOX inhibitor was not used in any of the fungicide sensitivity assays because the AOX inhibitors had significant effects on mycelial growth. One isolate from each collection site was randomly selected to test the effects of strobilurin fungicides. Serial dilutions of commercial Quadris™ (250 FL, Syngenta), Headline™ (250 FL, BASF), and Gem™ (500 SC, Bayer CropScience) were made in sterile double-distilled water to achieve active ingredient concentrations 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 μg ml^{-1} for azoxystrobin while 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, and 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 μg ml^{-1} were selected for pyraclostrobin and trifloxystrobin, respectively. Different fungicide concentrations were used for each of the three fungicides based on preliminary sensitivity ranges tested on a subset of the isolate population. As in the AOX inhibitor assay, stock fungicide solutions were added to molten PDA after autoclaving and cooling, and a 5.4 mm diameter agar plug was placed in the center of the plate for

incubation at 25°C in the dark for 28 hours. Two replicate plates were used for each isolate-fungicide concentration combination, and image analysis was conducted in Assess as per the AOX inhibitor assay. For each fungicide, the experiment was conducted twice.

Colony diameters were normalized by dividing each mean diameter by the mean diameter of the unamended PDA control plates after subtracting the plug diameter (5.4 mm). The NLIN procedure in SAS statistical software was used to analyze the nonlinear dose response curve of normalized colony diameter versus $\log_{10}$-transformed fungicide concentration for each isolate. A general, three parameter logistic equation of

$$Y = Min + \frac{Max - Min}{1 + 10\text{^}(X - log_{10}EC_{50})}$$

was fitted to determine the EC_{50}, the effective concentration of fungicide (ug ml^{-1}) that inhibits the colony growth 50% between the maximum and minimum colony diameter. Starting values of -1, 0, and 1 were supplied to the NLIN procedure for $\log_{10}EC_{50}$, Min, and Max values respectively.

To determine if isolates differed from the sensitive and resistant control isolates, ANOVA was conducted on the $\log_{10}$ EC_{50} values using the MIXED procedure of SAS software, with the fungicide, isolate, and the interaction as fixed effects and experiment within fungicide as a random effect. Significance was evaluated at α=0.05 using Fisher's least significant difference for Fungicide*Isolate groups. Isolates were considered resistant if their EC_{50} values were not significantly different from the resistant control isolate within each fungicide, sensitive if they were not different from or more sensitive than the sensitive control, or intermediate if they were significantly different than the two control isolates or grouped with both controls. Fungicide means were separated using α=0.05 and Bonferroni's correction. Assumptions of residual normality and homogeneity of variance were checked with the Shapiro-Wilk test in PROC UNIVARIATE and the 'group=' option in the random statement of PROC MIXED, respectively.

2.2.5 LIQUID MEDIUM FUNGICIDE SENSITIVITY ASSAYS. In order to test more isolates more quickly using less materials and labor, a 96-well microtiter assay was developed.

This assay could reasonably accommodate multiple isolates from the 40 sampled fields, unlike the solid medium assay. The number of isolates successfully collected from each field ranged from one to eight (average of four), and all 162 isolates were tested using the liquid medium assay. As in the amended solid medium assay, serial dilutions were made of Quadris™, Headline™, and Gem™, but diluted in potato dextrose broth (PDB, 24 g powder liter^{-1}) with a broader concentration range: 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 μg ml^{-1} for azoxystrobin; 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 μg ml^{-1} for pyraclostrobin; and 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 for μg ml^{-1} trifloxystrobin. A blank well (200 μl of unamended PDB), two control wells (150 μl of unamended PDB), and nine wells containing one of the fungicide concentrations above (150 μl of fungicide-amended PDB) were prepared for each isolate-fungicide combination in clear 96-well microtiter plates.

Mycelia served as inoculum in the form of six agar plugs (4-mm-diameter) taken from the growing margin of each isolate. The bottom half of the plug (away from the mycelial surface) was removed using a flamed scalpel, and the plugs were placed inside a 1.5 ml microcentrifuge tube containing 500 μl of unamended PDB. Plugs were comminuted by hand using mated plastic pestles. The mycelial suspension was then transferred to a 15 ml centrifuge tube containing 5.5 ml PDB with repeated pipetting in the 1000 μl pipet tip to aid in homogenization. For each isolate, 50 μl aliquots of mycelial suspension were added to the appropriate wells for a total volume of 200 μl in each well. This final well volume accurately reflected the fungicide concentrations previously stated. Triplicate wells on separate plates were used for each isolate-concentration combination. The plates were sealed with specialized adhesive plastic, and initial absorbance measurements were taken using a 96-well plate reader. After incubating for 48 hours in the dark at 25°C, a second absorbance measurement was taken. For the second absorbance reading, the plastic seals were replaced to remove overgrown mycelia and to ensure that all absorbance values reflected growth of the liquid-suspended mycelia only. The experiment was conducted twice.

Relative growth (RG) was calculated for each fungicide-amended well using the equation

$$RG = \frac{A_{48} - A_0}{U_{48} - U_0}$$

where A_0 and A_{48} represent the absorbance values for the inoculated, fungicide-amended wells at the initial and 48 hour readings, respectively, and U_0 and U_{48} for the inoculated, unamended control wells at the same time points (Seyran, Brenneman, and Stevenson 2010). Regression on RG versus $\log_{10}$ fungicide concentration was conducted for each row on each microtiter plate, yielding six EC_{50} values for each fungicide-isolate combination after both experiments were conducted. The same three parameter logistic equation and starting values were used as in the solid medium assay. The ANOVA was conducted using the MIXED procedure in SAS with a fixed effects of isolate, fungicide, and the interaction. The individual microtiter plates within each experiment served as a blocking variable. Tukey's adjustment was used to compare Fungicide*Isolate groups at the α=0.05 level, while Bonferroni's adjustment separated Fungicide groups (α=0.05). Testing of residuals was conducted again with SAS's PROC UNIVARIATE to assess normality using the Kolmogorov-Smirnov test, but testing homogeneity of variance was more complicated. Due to the large number of isolate groups, using the 'group=' option in the random statement could not be completed successfully. Instead, the Bartlett test was used in PROC GLM. As Bartlett's test is used for one-way ANOVA's, analysis was done separately for the effect of Isolate, Fungicide, and Fungicide*Isolate.

To compare the EC_{50} values between the solid and liquid medium assays, simple linear regression was used to analyze responses from the 40 isolates present in both assays. PROC REG in SAS was used on the $\log_{10}EC_{50}$ values generated from the solid and liquid medium assays. PROC UNIVARIATE was used to assess regression residuals for normality, and residuals were plotted against predicted values to check for homoscedasticity using PROC PLOT.

2.3 RESULTS

2.3.1 ISOLATE VERIFICATION. Initially, 39 of the 40 isolates selected for the solid medium assay amplified appropriately, indicating these isolates did indeed belong to AG I-1A

(Figure 2.3). One isolate failed to amplify, and a different random isolate was selected that amplified as expected. These results were corroborated by traditional anastomosis tester

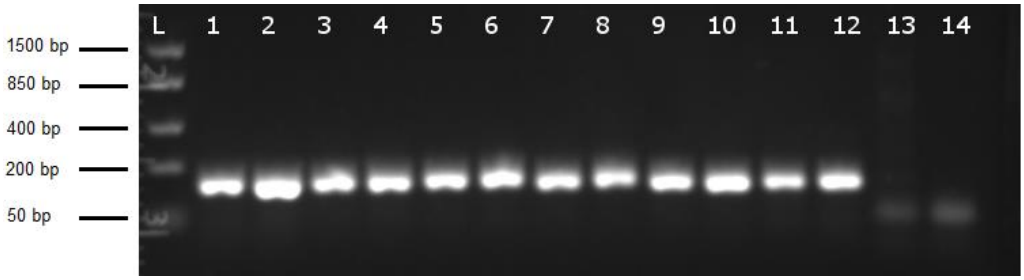

Figure 2.3. Typical results from the PCR assay to verify isolate anastomosis group. Lane L, DNA ladder. Lanes 1-11, positive result from collection site isolates. Lane 12, positive control. Lane 13, negative control (*Colletotricum fructivorum*). Lane 14, water control.

isolate classification, with the aberrant isolate being classified as a binucleate *Rhizoctonia* sp. Similarly, all other isolates used for the liquid medium assay amplified as expected, for all 162 isolates of *R. solani* AG 1-IA used in this study.

2.3.2 ALTERNATIVE OXIDASE INHIBITOR TOXICITY ASSAYS. Both AOX inhibitors had demonstrable effects on mycelial growth. For PG, the chemical concentration effect was highly significant at P = < 0.0001, while neither the effect of isolate nor the interaction was significant at P = 0.2028 and P = 0.0583, respectively. Therefore, the results for the five isolates were combined for the analysis. Concentrations of 100 and 150 μg PG ml^{-1} had significantly different means from the unamended control treatment, but the 50 μg ml^{-1} concentration did not after Dunnett's adjustment (Figure 2.4).

The chemical concentration (P = <0.0001) was highly significant in the SHAM trial, and the effect of isolate (P = 0.2035) and the interaction (P = 0.2751) were again not significant. All three concentrations of SHAM tested were significantly different than the control based on Dunnett's adjustment (Figure 2.4).

2.3.3 SOLID MEDIUM FUNGICIDE SENSITIVITY ASSAYS. Analysis of variance was conducted on $\log_{10}EC_{50}$ values to uphold assumptions of normality. The effect of experiment was not significant (P = >0.05) so experiments were combined. The effects of fungicide, isolate,

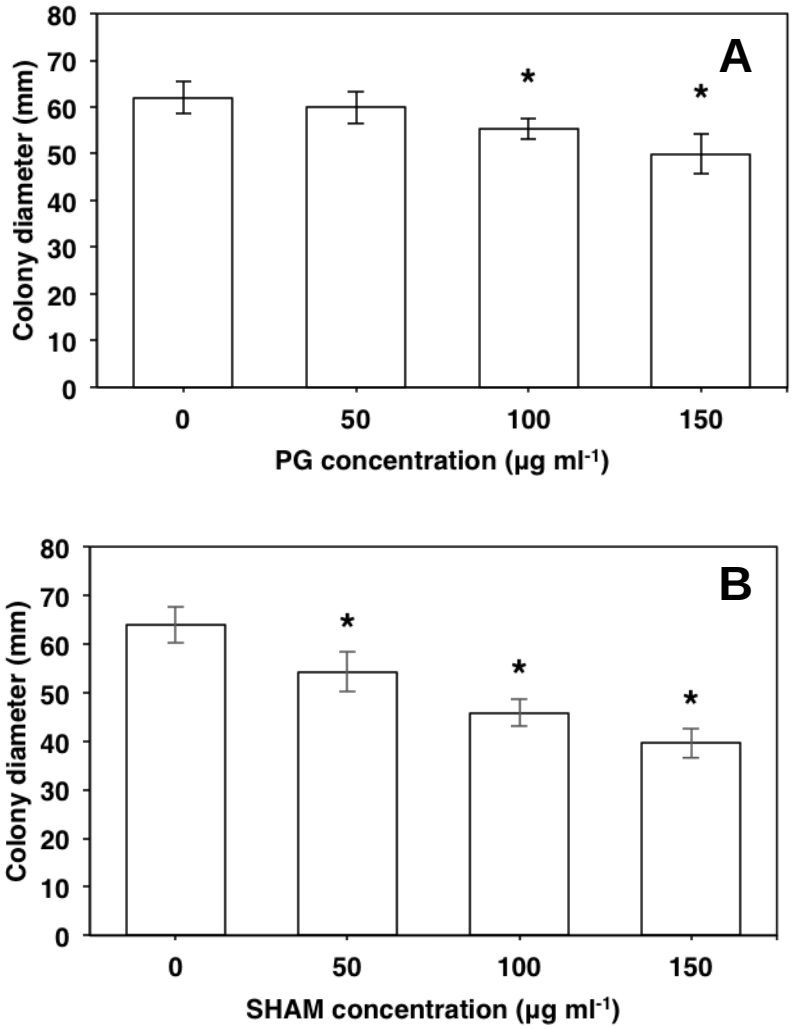

Figure 2.4. Colony growth as affected by alternative oxidase inhibitors. A, propyl gallate (PG); B, salicylhydroxamic acid (SHAM). Columns marked with an asterisk (*) are significantly different than the zero-concentration control at α=0.05 based on Dunnett's adjustment. Error bars represent 95% confidence intervals based on the mean.

and the interaction were significant (Table 2.1). For each fungicide, EC_{50} value means were back-transformed and reported in the experimental concentration (Table 2.2). The pyraclostrobin treatment had a greater mean (0.0642 µg ml^{-1}) than those for azoxystrobin (0.0587 µg ml^{-1}) and trifloxystrobin (0.0061 µg ml^{-1}) in the solid medium assays. Pyraclostrobin grouped with azoxystrobin as not significantly different, while both means were significantly higher than the trifloxystrobin treatment mean.

Table 2.1. Main and interaction effects in analysis of variance for both solid and liquid medium strobilurin sensitivity assays.

	Solid medium			Liquid medium		
Effect	d.f.	*F*	*P*	d.f.	*F*	*P*
Fungicide	2	114.51	0.0015	2	2603.63	<0.0001
Isolate	39	4.32	<0.0001	161	60.73	<0.0001
Fungicide x Isolate	78	3.38	<0.0001	322	18.31	<0.0001

Table 2.2. Treatment means of effective concentrations that inhibited mycelial growth to 50% between the maximum and minimum responses (EC_{50}) for tested fungicides in both solid and liquid medium strobilurin sensitivity assays.

	EC_{50} (μg ml^{-1})[z]	
Fungicide	Solid medium	Liquid medium
Headline™ (pyraclostrobin)	0.0642 a	0.0341 a
Quadris™ (azoxystrobin)	0.0587 a	0.0106 b
Gem™ (trifloxystrobin)	0.0061 b	0.0035 c

[z] Means in the same column marked with the same letter are not significantly different after Bonferroni adjustment (α=0.05).

The azoxystrobin EC_{50} values (averaged across experiments for each isolate and back transformed) were log-normally distributed (W= 0.9858, P= 0.5241 for the $\log_{10}EC_{50}$ value distribution), had a median of 0.0486 μg azoxystrobin ml^{-1}, and ranged from 0.0029 to 0.6352 μg ml^{-1} (Figure 2.5). The distribution was weakly bimodal with positive skewness towards higher EC_{50} values (and consequently lower sensitivity). As expected, the resistant and sensitive control isolates had significantly different EC_{50} values for azoxystrobin. An isolate was considered resistant if it shared a significance group with the resistant control only, sensitive if it shared a significance group with the sensitive control only, and intermediate if it matched both controls. Out of the 40 isolates tested, each representing a collection site, 11 were resistant (27.5%), three were intermediate (7.5%), and 26 were sensitive (65%, including six isolates significantly more sensitive than the sensitive control). Six resistant isolates clustered

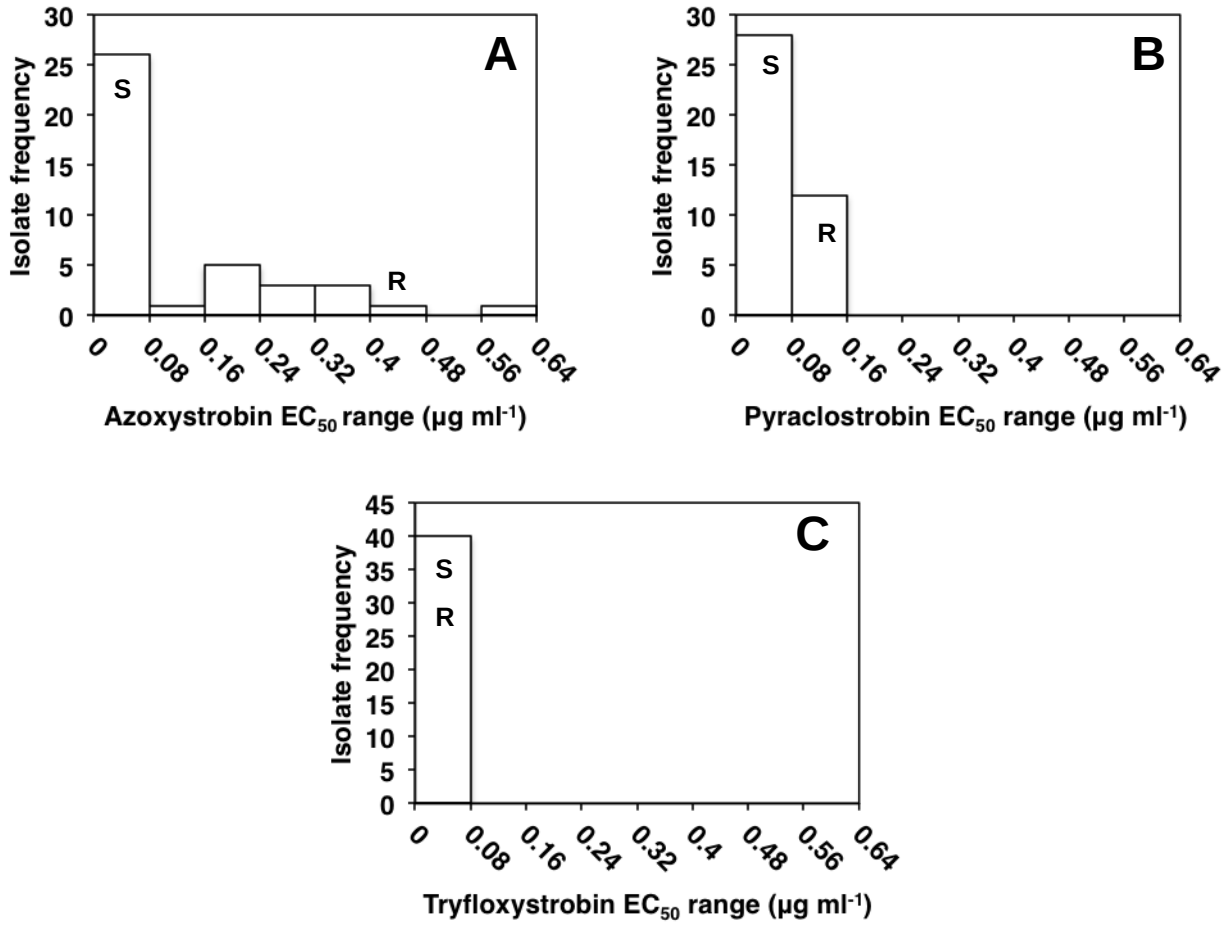

Figure 2.5. Distributions of effective concentrations to inhibit mycelial growth by 50% (EC_{50}, n=40 isolates) using the solid medium assay. R and S mark the bins that contain the resistant (to azoxystrobin) and sensitive control isolates, respectively. A, azoxystrobin; B, pyraclostrobin; C, trifloxystrobin.

near the resistance origin, along with the three intermediate isolates (Figure 2.6). However, two resistant isolates were located more than 40 km away from the resistance origin.

Pyraclostrobin EC_{50} values were sufficiently normally distributed (W=0.9585, P= 0.1490), with a range from 0.0213 to 0.1547 µg pyraclostrobin ml^{-1} with a median of 0.0712 µg ml^{-1} (Figure 2.5). The distribution showed slight positive skewness. The resistant and sensitive control isolates had significantly different EC_{50} values for pyraclostrobin. There were three isolates (7.5%) that grouped with the resistant control isolate (field-resistant to azoxystrobin), 28 intermediate isolates (70%) that grouped with both the resistant and sensitive control, and nine isolates (22.5%) that grouped only with the sensitive isolate indicating sensitivity. The

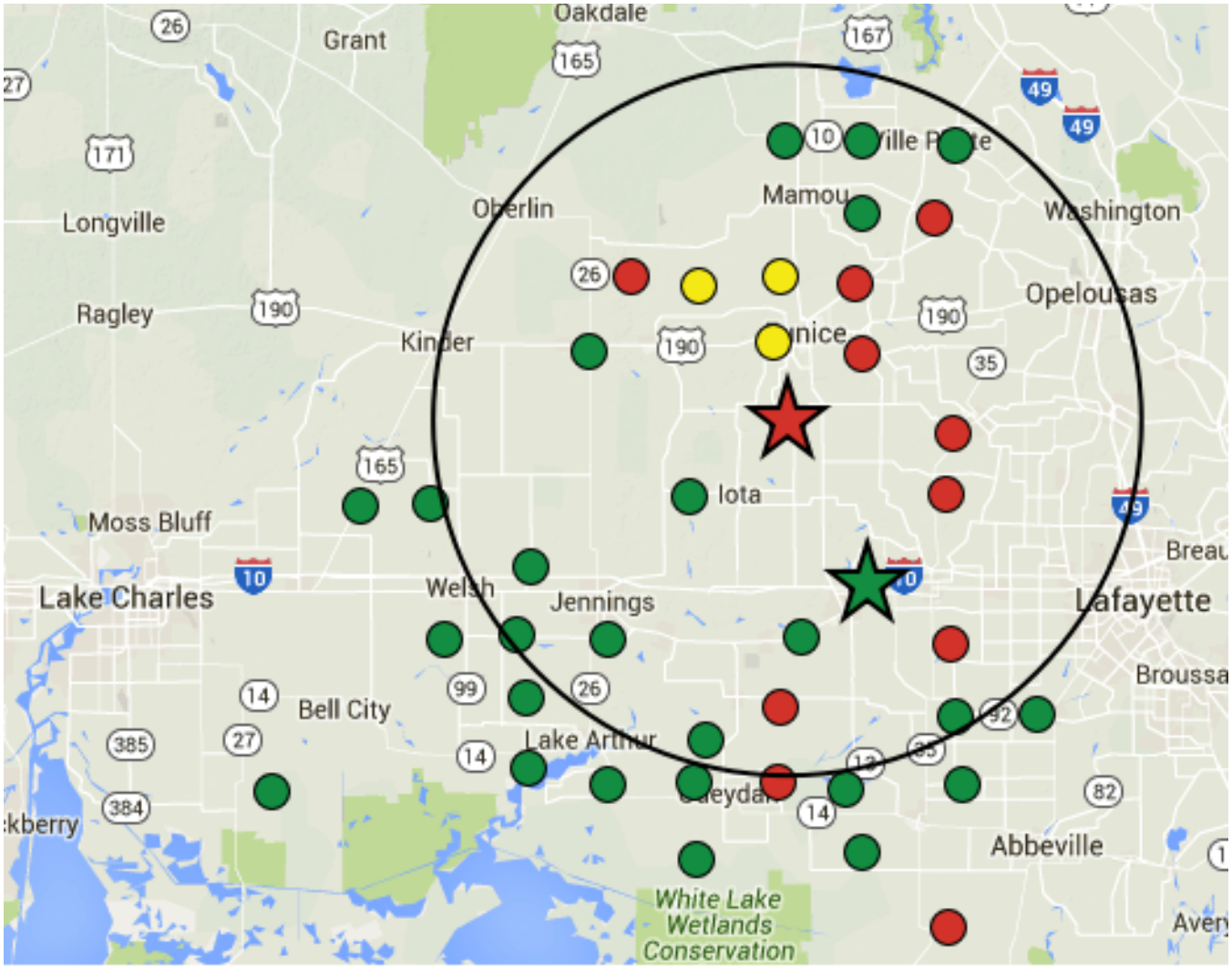

Figure 2.6. Isolate locations representing azoxystrobin sensitivity reaction. Colored circles mark fields where isolates were collected. Green coloration indicates sensitive response, yellow indicates intermediate, and red, resistant. Red star marks resistance origin, and black circle marks the 40 km radius from this area. Green star marks sensitive isolate collection field.

resistant isolates were clustered around the resistance origin, while the intermediate isolates were more widely distributed, with 14 outside the 40 km range from the resistance origin (Figure 2.7).

Trifloxystrobin EC_{50} values only ranged from 0.0016 to 0.0209 µg trifloxystrobin ml^{-1}, had a median of 0.0057 µg ml^{-1}, and were log-normally distributed (W=0.9848, P= 0.4658 for $log_{10}EC_{50}$ distribution) (Figure 2.5). The azoxystrobin-resistant control isolate grouped with the strobilurin-sensitive control isolate as not significantly different. This lack of clear differences in EC_{50} values across the control isolates and narrow range prevented sensitivity categorization

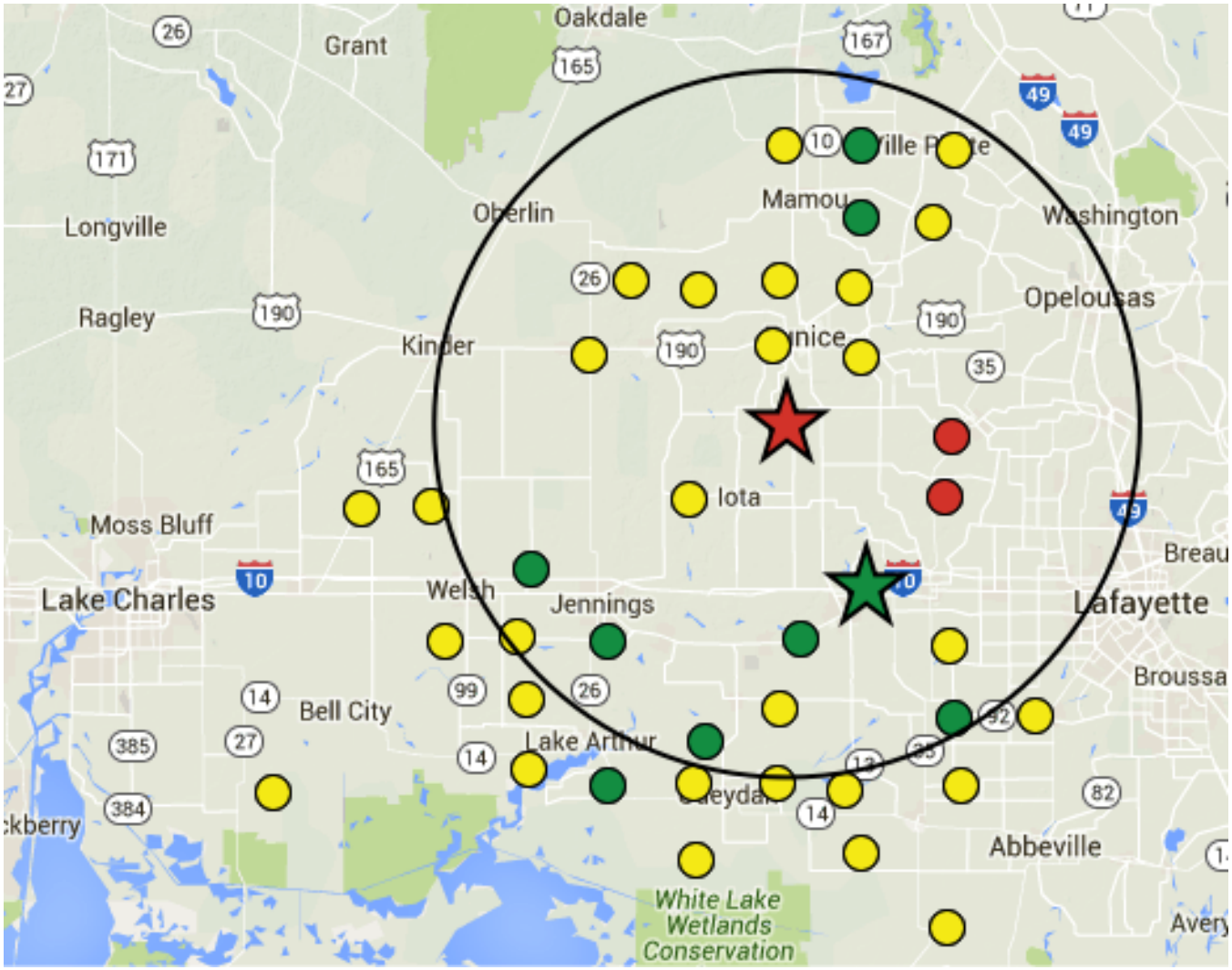

Figure 2.7. Isolate locations representing pyraclostrobin sensitivity reaction. Colored circles mark field where isolates were collected. Green coloration indicates sensitive response, yellow indicates intermediate, and red, resistant. Red star marks resistance origin, and black circle shows 40 km radius from this area. Green star marks sensitive isolate collection field.

in the trifloxystobin assay, possibly indicating sensitivity across the isolate population.

2.3.4 LIQUID MEDIUM FUNGICIDE SENSITIVITY ASSAYS. The number of isolates successfully collected from each field ranged from one to eight (average of four). Because the effect of Isolate was found to be homoscedastic based on Bartlett's test while Fungicide and Isolate by Fungicide were heteroscedastic, the ANOVA model was fitted using a 'group=Isolate' option in the random statement of PROC MIXED. The effects of Fungicide, Isolate, and Fungicide by Isolate were all significant in the liquid medium assay (Table 2.1). Unlike in the solid medium assay, all three fungicide treatment means were significantly

different. The means retained the same relative order, however, with pyraclostrobin having the highest mean (0.0341 μg ml^{-1}) and trifloxystrobin having the lowest (0.0035 μg ml^{-1}) (Table 2.2).

The azoxystrobin EC_{50} values ranged from 0.0006 to 0.2923 μg ml^{-1}, with a median of 0.0024 μg ml^{-1}. The distribution was strongly bimodal (modes within 0 to 0.015 and 0.09 to 0.105 μg ml^{-1}) and was heavily skewed towards higher EC_{50} values (Figure 2.8). The EC_{50} values of the resistant and sensitive control isolates were significantly different. Significantly grouping with the resistant control, 63 isolates (38.9%) tested resistant to azoxystrobin, while 91 (56.2%) were sensitive including the sensitive control, leaving eight intermediate isolates (4.9%) that were significantly different than both controls. Resistant isolates were identified in 22 of the 40 collection sites (55%), sensitive isolates were found in 30 sites (75%), and intermediate in 8 (20%) (Table 2.3). Eight and 15 sites contained sampled isolates that were only resistant or only sensitive, respectively. Intermediate-response isolates were only found in conjunction with isolates from other sensitivity categories. Many of the resistant isolates were located near the resistance origin, but 20 were located greater than 40 km away distributed over seven collection sites (Figure 2.9). There were also three intermediate isolates (in three sites) beyond 40 km from the resistance origin.

For pyraclostrobin sensitivity using the liquid medium assay, EC_{50} values ranged from 0.0096 to 0.2534 μg ml^{-1}, having a median of 0.0245 μg ml^{-1} (Figure 2.8). The pyraclostrobin distribution was more weakly bimodal than the azoxystrobin distribution with less separation between the modes. The pyraclostrobin modes were located between 0.013 to 0.026 and 0.052 to 0.065 μg ml^{-1}. This distribution also displayed extreme high EC_{50} values causing positive skewness. The EC_{50} values of the resistant and sensitive control isolates did not significantly differ in the pyraclostrobin assay, preventing explicit sensitivity categorization of the isolates. Only one isolate out of 162 differed significantly from the resistant control, while 14 differed from the sensitive control.

The distribution of trifloxystrobin sensitivity responses was more unimodal than the other two fungicides, with a prominent mode between 0.003-0.00375 μg ml^{-1} and a possible

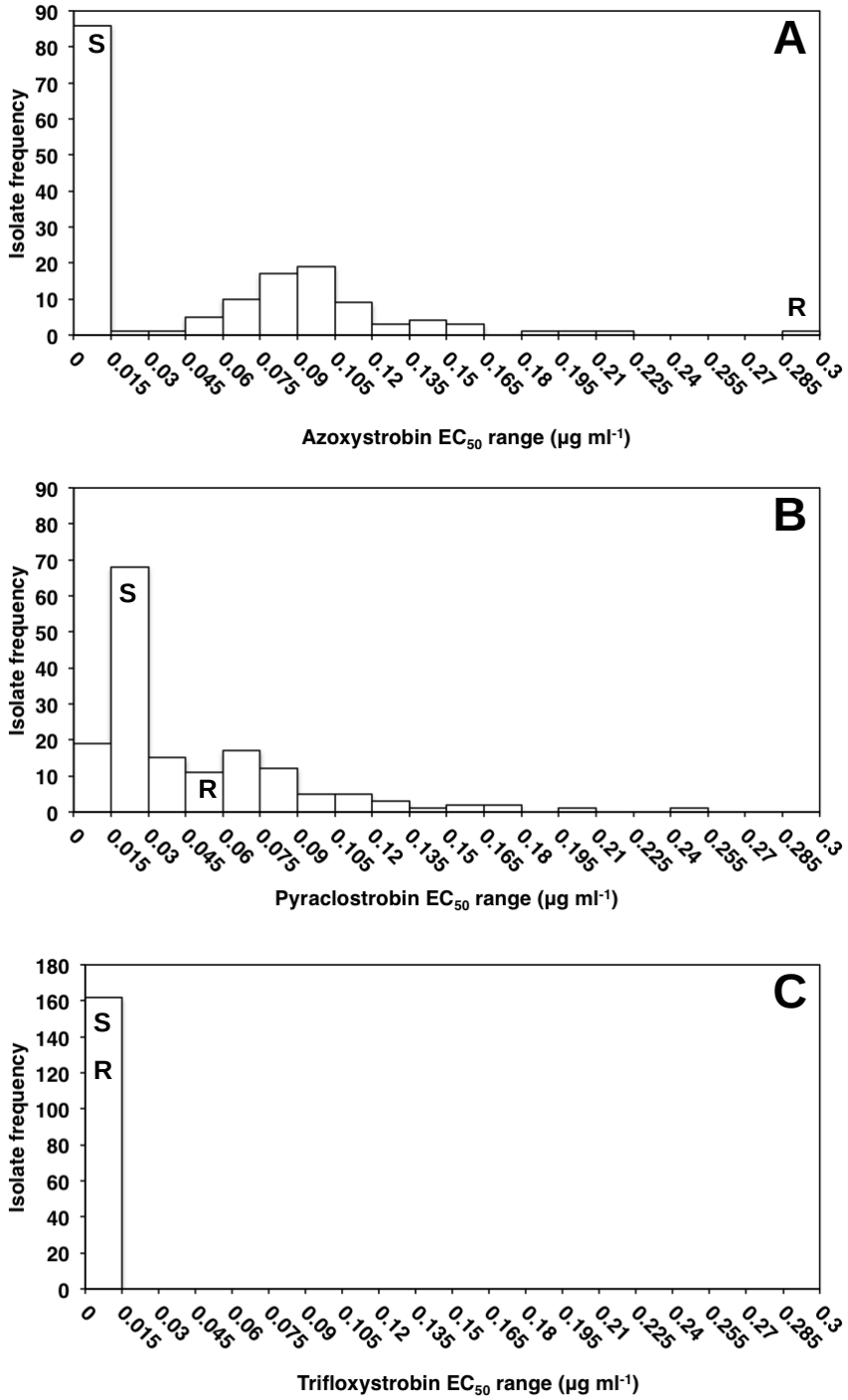

Figure 2.8. Distributions of effective concentrations to inhibit mycelial growth by 50% (EC_{50}, n=162 isolates) using the liquid medium assay. R and S mark the bins that contain the resistant (to azoxystrobin) and sensitive control isolates, respectively. A, azoxystrobin; B, pyraclostrobin; C, trifloxystrobin.

Table 2.3. Azoxystrobin sensitivity breakdown of isolates in the 40 collection sites based on the liquid medium assay.

Collection site[z]	Resistant	Intermed-iate	Sensitive	Collection site	Resistant	Intermed-iate	Sensitive
1*	3	0	2	21*	2	0	3
2*	0	0	2	22	0	0	6
3*	0	0	1	23	2	0	0
4*	0	1	4	24	4	0	4
5	0	0	6	25*	0	0	6
6	1	0	1	26*	1	0	2
7	2	1	2	27	0	0	7
8	0	1	5	28	3	0	2
9	0	0	2	29	1	0	0
10	5	1	0	30	1	0	3
11	0	0	1	31	3	0	0
12*	1	0	1	32	1	1	3
13*	2	0	4	33	1	0	0
14*	0	0	1	34	0	0	5
15*	8	0	0	35*	0	1	4
16*	0	0	5	36 (S)	0	0	1
17*	3	1	2	37 (R)	7	1	0
18	0	0	2	38	6	0	0
19	0	0	2	39	4	0	0
20*	0	0	2	40	2	0	0

[z] R represents the resistance origin and S represents the site where the sensitive control isolate was collected in 1972. Sites marked with an asterisk (*) are located more than 40 km away from the resistance origin.

second mode between 0.00825-0.009 μg ml^{-1}. This distribution was again positively skewed.

Ranging from 0.0011 to only 0.0127, the trifloxystrobin EC_{50} values decidedly had the smallest

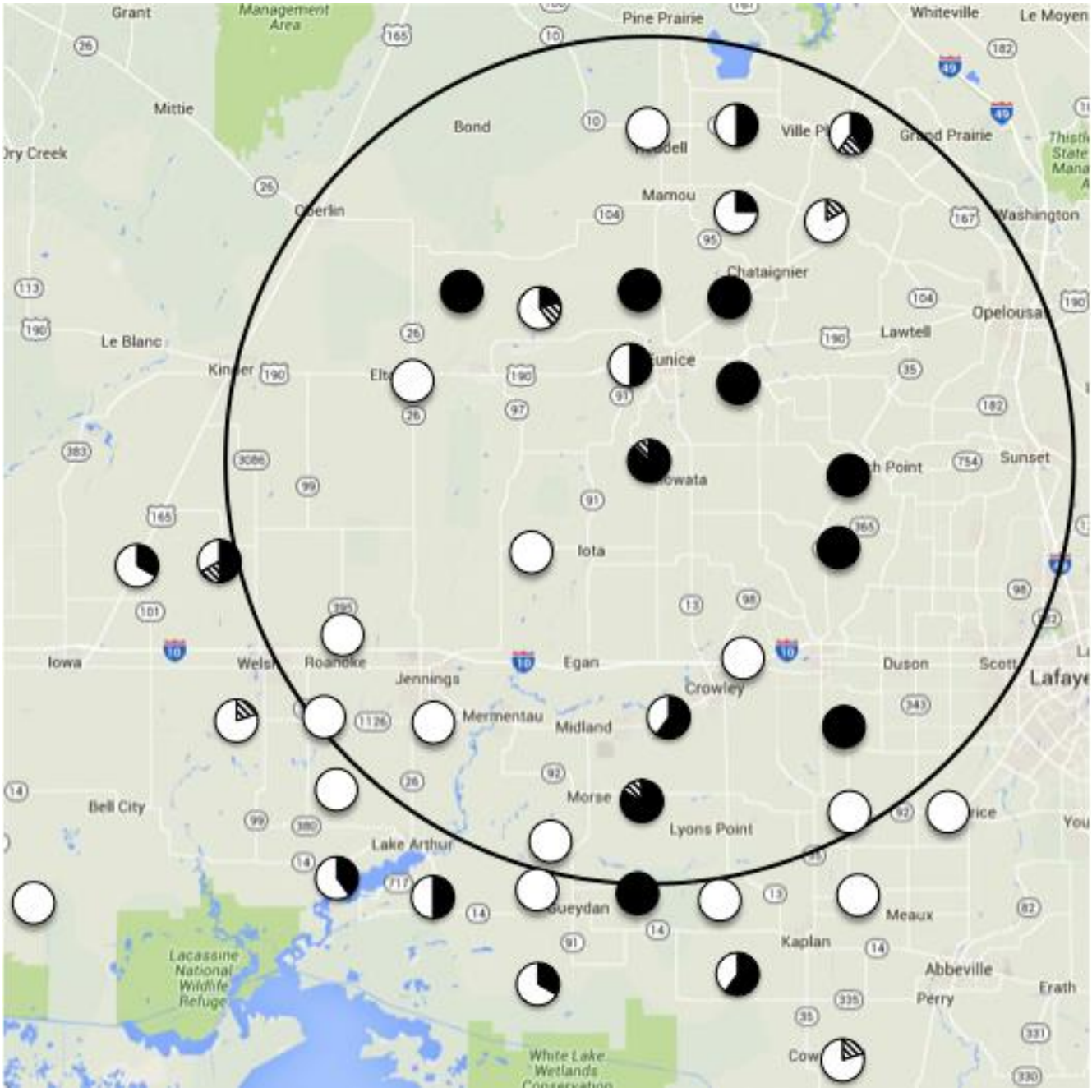

Figure 2.9. Map showing proportional breakdown of isolate sensitivity to azoxystrobin at each collection site as determined by the liquid medium assay. Black indicates resistant response, diagonal hatching indicates an intermediate response, and white is sensitive. The black circle marks a 40 km radius from the resistance origin, within which is the only area formerly known to have azoxystrobin-resistant isolates of *R. solani* in Louisiana.

range. The median was 0.0035 μg ml^{-1}. Almost 60% of the isolates (96/162) had EC_{50} values within the 0.003-0.00375 μg ml^{-1} mode or lower. The sensitive and resistant control isolates did not differ in the trifloxystrobin assay, again precluding categorization of isolate sensitivity to

this fungicide. Only four isolates out of 162 had significantly different EC_{50} values than the resistant control, and only three differed from the sensitive control.

The correlation of the solid and liquid medium $\log_{10}EC_{50}$ values varied by fungicide for the 40 isolates of *R. solani* (Figure 2.10). The $\log_{10}EC_{50}$ values were strongly correlated for azoxystrobin. The regression was significant (P = <0.0001), with an R^2 value of 0.7611 and a slope of 1.36 $\log_{10}(\mu g\ ml^{-1})$. The pyraclostrobin regression was also significant (P = 0.0066), but R^2 was much lower at 0.1784, with a slope of 0.55 $\log_{10}(\mu g\ ml^{-1})$. For trifloxystrobin, however, the regression was not significant (P = 0.5740).

2.4 DISCUSSION

Testing strobilurin sensitivity is often conducted with the addition of AOX inhibitors (Amiri, Heath, and Peres 2013; Wood and Hollomon 2003; Zhang 2012) to mimic *in vivo* conditions where plant flavones are thought to suppress AOX activity (Mizutani 1996; Olaya, Zheng, and Köller 1998). However, in this study, AOX inhibitors were toxic to mycelial growth of five isolates of *R. solani* AG 1-IA *in vitro*. Adding 20 μg SHAM ml^{-1} reduced pyraclostrobin EC_{50} values during both *in vitro* and *in planta* assays using *Sclerotinia sclerotiorum*, prompting the authors to conclude that SHAM should not be added to pyraclostrobin *in vitro* assays with this pathogen due to SHAM deflating the true EC_{50} value (Liang et al. 2015). Toxicity *in vitro* has been found with other inhibitor-pathogen systems. Up to 60% reduction in mycelial growth of *Magnaporthe oryzae* was found when 150 μg SHAM ml^{-1} was added to azoxystrobin *in vitro* assays (Kunova et al. 2013), compared with 38% inhibition in our *R. solani*-SHAM assays at the same concentration. Toxicity of PG and SHAM was shown for *Fusicladium effusum* in both liquid and solid media (Seyran, Brenneman, and Stevenson 2010). A concentration of 1 mM PG (212 μg ml^{-1}) alone inhibited colony growth of 11 *Botrytis cinerea* isolates more than a combination of azoxystrobin and PG or azoxystrobin alone (Ishii et al. 2009).

These inhibitors also lack uniform effects across fungicides and isolates of interest. Addition of an AOX inhibitor may not affect EC_{50} values of a certain fungicide but alter that of another, as found in the case of unaffected azoxystrobin EC_{50} values but altered pyraclostrobin

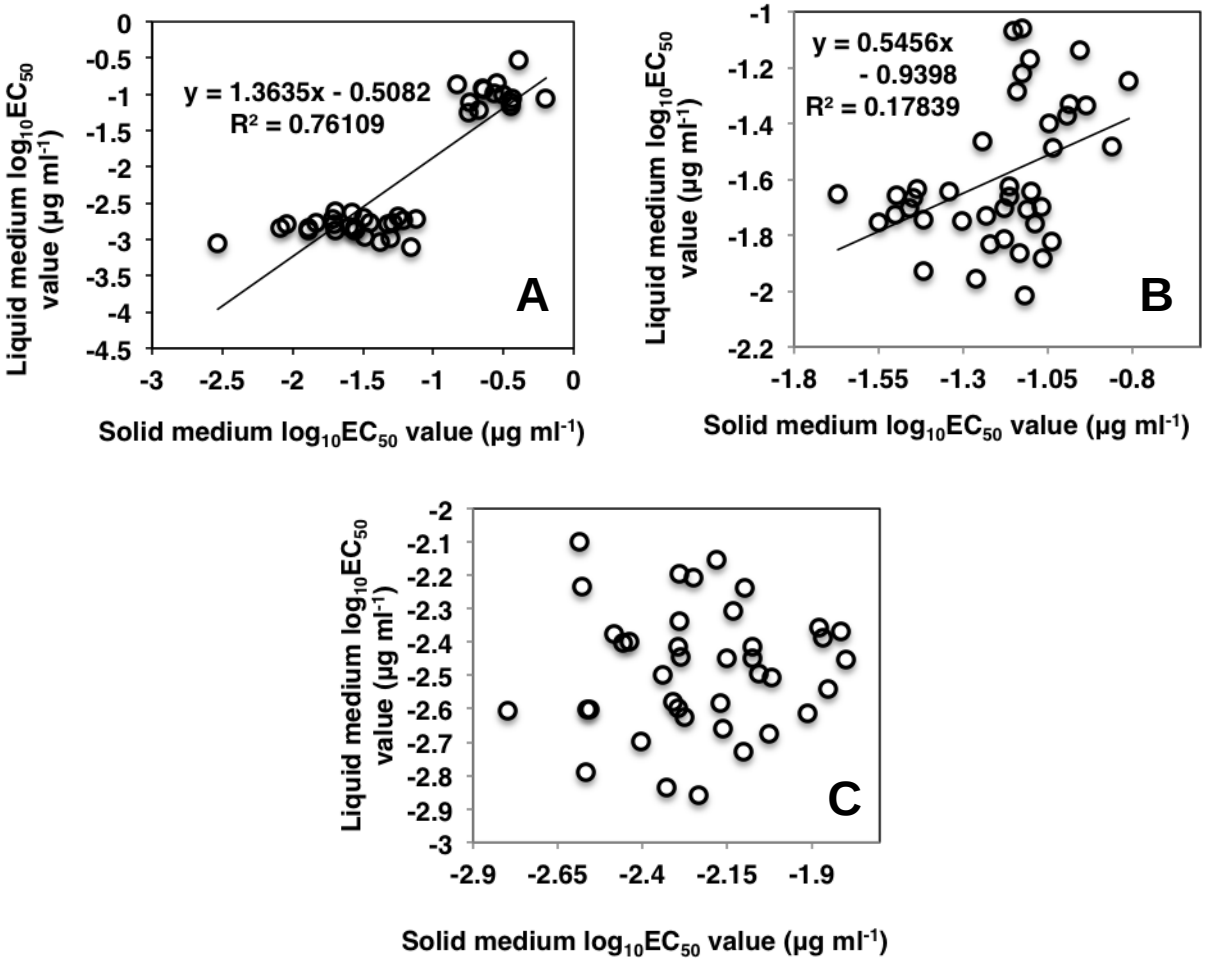

Figure 2.10. Correlation plots of $log_{10}EC_{50}$ values derived from the solid and liquid medium assays for each of three fungicides tested. If the regression showed a significant improvement over a slope of 0, the regression equation and R^2 value are shown. A, azoxystrobin; B, pyraclostrobin; C, trifloxystrobin.

EC_{50} values when testing *Guignardia citricarpa* with the addition of SHAM (Hincapie et al. 2014). Isolates of *Alternaria alternata* were differentially affected by SHAM (Vega et al. 2012). The five isolates of *R. solani* AG 1-IA were not differentially affected by AOX inhibitors in this study, but other isolates may have different responses. AOX inhibitors were not suitable for use in *R. solani* AG 1-IA *in vitro* strobilurin sensitivity assays because of confirmed toxicity.

The correlation of solid medium strobilurin sensitivity assay results with liquid medium results exhibited variability among the three fungicides evaluated. The azoxystrobin $log_{10}EC_{50}$ values were highly correlated between both assays ($R^2 = 0.76$), indicating the liquid medium assay is a suitable replacement for the more time- and resource-intensive solid

medium assay. Correlations as high as $R^2 = 0.962$ (Rampersad 2011) and 0.9943 (Cox et al. 2009) were found between growth inhibition of solid and liquid medium assays using resazurin dye, though EC_{50} value correlations were not reported. Media preparation for the solid medium assay reported herein requires five work days, while liquid medium requires only two. Using the liquid assay, 24 isolates were evaluated in triplicate at once, across three fungicides. Comparatively, 40 isolates in duplicate for only one fungicide at a time could be evaluated using the traditional solid medium assay. Digital measurement of the solid medium photographs can take a week or more, while the liquid medium data is ready for regression analysis immediately.

An interchangeable relationship was less supported for the two methods in pyraclostrobin and trifloxystrobin trials because the liquid assay $\log_{10}EC_{50}$ values were not strongly related to the solid medium $\log_{10}EC_{50}$ values for these two fungicides. The strength of correlation between liquid and solid assays varied among fungicides also for *Verticillium dahliae* (Rampersad 2011). Within the solid medium results presented herein, the pyraclostrobin values did not contain large differences, resulting in many intermediate sensitivity ratings, and the trifloxystrobin assay did not show a difference even between the sensitive and resistant control. This absence of distinct differences among isolates also held true in the liquid medium results and could help explain the lack of correlation between the solid and liquid medium assays. If there are no distinct differences among the isolates, the assays may fail to predict EC_{50} values accurately due to isolate similarities. However, the EC_{50} value distributions for each fungicide were similar in both the solid and liquid medium assays, suggesting a similar trend in isolate population sensitivity regardless of assay type used. For this reason, if a 96-well plate reader is accessible, the liquid medium assay described herein can be used for high-throughput processing of *R. solani* samples for *in vitro* strobilurin sensitivity testing, especially if azoxystrobin is the fungicide of interest. The liquid medium assay also revealed a broader range of pyraclostrobin sensitivity, rivaling the range of the azoxystrobin sensitivity distribution, whereas the solid medium assay reported a restricted pyraclostrobin sensitivity range. This increased sensitiveness combined with the added depth

of information provided with the ability to identify multiple isolate sensitivity-types in one collection site make the liquid medium assay preferable for a more complete pathogen sensitivity picture.

The nonlinear regression method used herein to determine EC_{50} values did not require growth parameters, such as colony diameter or light absorbance (in this study), to fall below half the unamended value. For example, if the colony diameter of the unamended PDA plate measured 100 mm, EC_{50} could still be determined if the diameters of the fungicide-amended plates ranged from 60 to 100 mm by evaluating the dose response curve. This method relies on growth plateaus at the lowest and highest fungicide concentrations tested rather than complete growth inhibition. In fact, the fungicides tested failed to completely inhibit mycelial growth at the concentrations tested as evidenced by non-zero growth parameters at the highest concentrations used (data not shown). This method of EC_{50} determination allows isolates from different sensitivity ranges to be tested using a relatively small range of fungicide concentrations. The protocol also allows more specific EC_{50} values to be assigned in the absence of reaching half the unamended growth parameter if growth plateaus are demonstrated. Azoxystrobin EC_{50} values were found to be 20 or > 30 μg ml^{-1} in four isolates of *R. solani* AG 3 (potato pathogen) using a solid medium assay (Djébali et al. 2014), which are about 30 fold or greater than the highest azoxystrobin EC_{50} value on solid medium of 0.6352 μg ml^{-1} presented herein. These high EC_{50} values could be partially attributed to the fungicides failing to reduce growth parameters by half.

An EC_{50} of 0.008 μg azoxystrobin ml^{-1} on solid medium was reported for an *R. solani* isolate of unknown AG (Jin et al. 2009), which falls within the azoxystrobin EC_{50} values of the AG 1-IA isolates tested. Sugarbeet isolates of *R. solani* AG 2-2 were tested with a solid medium assay, and contrary to our results, pyraclostrobin was found to have the lowest EC_{50} value (means of 0.3 and 0.6 μg ml^{-1} for baseline and non-baseline isolates) and trifloxystrobin the highest (97.1 and 341.7 μg ml^{-1}), with azoxystrobin falling between (4.8 and 296.0 μg ml^{-1}) (Arabiat and Khan 2014).

The distribution of EC_{50} values across a sampled pathogen population can illustrate sensitivity shifts. For example, one instance of benomyl resistance was made apparent by field level management problems of eyespot disease of cereals and also by the increasingly bimodal distribution of numeric sensitivity ratings across isolates of *Oculimacula yallundae* and *O. acuformis* (Parnell et al. 2008). Methyl benzimidazole carbamates, such as benomyl, are single action site fungicides like strobilurins. This increases the risk of fungicide resistance development as pathogen populations quickly accumulate the necessary point mutations to avoid fungicide activity, manifesting as qualitative resistance. Fungal isolates that have developed qualitative resistance can have distinctly higher EC_{50} values than sensitive isolates, creating a bimodal distribution. The distribution of azoxystrobin responses was strongly bimodal. The azoxystrobin assays benefited from inclusion of a known field-resistant isolate, and thus the resulting sensitivity categories are reasonably reliable. While the field-level pyraclostrobin and trifloxystrobin sensitivity of our resistant control isolate is unknown, it can be inferred that resistance to pyraclostrobin is developing among the *R. solani* population in southwestern Louisiana due to the bimodal distribution of sensitivity responses and positive skew apparent in the liquid medium results. Accordingly, resistance to trifloxystrobin is not supported by these results due to the tightly clustered distribution.

Cross-resistance, then, is more strongly supported between azoxystrobin and pyraclostrobin than between azoxystrobin and trifloxystrobin in this case. While cross-resistance is often assumed for strobilurins, it is not always empirically supported. Isolates of *Alternaria solani* were shown to be resistant (could grow on solid media amended with 507 μg ml^{-1}) to azoxystrobin, pyraclostrobin, and trifloxystrobin; resistant to azoxystrobin and trifloxystrobin but sensitive (could not grow on fungicide-amended solid media) to pyraclostrobin; or resistant to only azoxystrobin (Fairchild, Miles, and Wharton 2013). Trifloxystrobin could still manage *Pyricularia grisea* with the F129L mutation at the field level while azoxystrobin could not (Vincelli and Dixon 2002), and pyraclostrobin generally resulted in increased yield compared to azoxystrobin when applied to *Pyrenophora teres* populations on barley also known to exhibit the F129L cytochrome b mutation (Semar et al. 2007). These

results suggest that trifloxystrobin might still be viable on azoxystrobin-resistant isolates of *R. solani* on rice, but field testing is required to determine if this is the case.

There are no publicly available mitochondrial genomes of *R. solani* AG 1-IA, and this makes rapid, inexpensive, genetic characterization of mitochondrial mutations difficult. Primers are being developed to amplify regions containing the codons for the 129, 137, and 143th amino acids in the cytochrome b gene to determine the presence or absence of known strobilurin resistance mutations at these sites.

A different molecular method was successfully used to confirm the identity of collected isolates. The described PCR procedure produced AG assignments in 100% agreement with the traditional anastomosis tester isolate method. This DNA procedure can be used to quickly check the identity of *R. solani* AG 1-IA isolates, especially among scientists that lack the technical expertise and tester isolates required by the traditional method.

Evidence is presented that there are isolates of *R. solani* resistant to azoxystrobin more than 40 km west, southwest, and south from the resistance origin. From this finding, it is concluded that the geographic distribution of azoxystrobin-resistant isolates is greater than previously known. Based on field results at the resistance origin, azoxystrobin applications will be highly ineffective for managing these resistant isolates. The mode of increased distribution is unknown. Resistant isolates could be disseminating to new fields via contaminated field equipment or crop debris, or resistance could be developing *de novo*. Genetic studies could address this question. It is also presently unknown if the azoxystrobin-resistant isolates display any sort of fitness cost compared to sensitive isolates, or if resistant isolate proportions remain unchanged in the pathogen population after fungicide selection pressure is removed.

There is also evidence of pyraclostrobin resistance in the tested population of *R. solani* as indicated by some significant EC_{50} differences among isolates in the solid medium assay and the bimodal and positively skewed EC_{50} distribution in the liquid medium assay. The low level of significant differences among isolate responses could be reevaluated if an isolate with field

resistance to pyraclostrobin could be included in the assays for comparison. Isolates with high pyraclostrobin EC_{50} values identified in this study could be potential candidate isolates.

Strobilurin resistance among the *R. solani* population causing sheath blight on rice will continue to be a problem in southwestern Louisiana. Evidence supports possible cross resistance between two fungicides but not a third. The isolates and results described herein will serve as a baseline for testing sensitivity to strobilurins and other fungicides, allow continual monitoring of fungicide efficacy, and guide management decisions for sheath blight on rice in Louisiana.

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CHAPTER 3. SUMMARY

In 1858, Julius Kühn gave the name *Rhizoctonia solani* to a plant pathogen causing disease on potatoes. *Rhizoctonia* means 'death to roots' and *solani* refers to the plant family that potatoes belong to. Today, a rice pathogen bearing the name *Rhizoctonia solani* causes damage to rice stems, leaves, and seeds via the disease sheath blight. Sheath blight is a major disease on rice grown in the southeastern United States causing grain loss annually. Many, if not most, rice fields have a history of this disease. The pathogen, *Rhizoctonia solani* AG 1-IA, has high overwintering ability as sclerotia and mycelia-infested crop debris. To reduce the damage caused by this disease, growers will typically apply a fungicide to their fields. An application of azoxystrobin is recommended for sheath blight management as reliable genetic resistance to sheath blight in the rice germplasm is presently lacking.

In 2011, the advent of fungicide resistance to azoxystrobin in *Rhizoctonia solani* AG 1-IA prompted fungicide sensitivity testing. A total of 162 *R. solani* isolates were collected from 40 rice production fields in southwestern Louisiana to assess sensitivity to azoxystrobin and also to trifloxystrobin (also labeled for rice) and pyraclostrobin (labeled for soybeans - *Rhizoctonia solani* AG 1-IA can also cause aerial blight on soybeans, and rice and soybeans are often rotated).

Collected isolates were checked for proper identity using PCR, and stored on frozen rye seed. The use of alternative oxidase inhibitors was rejected due to toxicity. A fungicide-amended solid medium assay was used to test one isolate from each of the 40 fields, and azoxystrobin resistance was clearly represented by large, significant differences between resistant and sensitive control isolates. Results for pyraclostrobin were less distinct, and even less differences among isolates were seen in trifloxystrobin sensitivities.

All 162 isolates were tested with a higher-throughput fungicide-amended liquid medium assay, which supported strong isolate differences in azoxystrobin responses. Strong significant differences were not found among isolate responses to pyraclostrobin or trifloxystrobin. However, the sensitivity distributions in both the solid and liquid medium assays indicate resistance to azoxystrobin and emerging resistance to pyraclostrobin among *R.*

solani in southwestern Louisiana. The liquid medium assay increased isolate throughput and provided insight into within-field sensitivity variation.

Azoxystrobin resistance was found in a greater geographic area than reported previously, and azoxystrobin management of sheath blight is predicted to be highly ineffective in these areas. Evidence for developing pyraclostrobin resistance was also demonstrated. Trifloxystrobin could potentially remain effective on azoxystrobin- or pyraclostrobin-resistant isolates of R. *solani* on rice. This information can be used to develop new fungicide rotations for rice disease management.

Continued monitoring and field-testing of pathogen populations will inform management recommendations for sheath blight in southwestern Louisiana. The isolates, information, and protocols recorded herein can serve as baselines and guides for future studies.

VITA

Allysson Gayle Lunos was born in the snows of Michigan, raised in the heat of Texas, and calls the rainy city of Baton Rouge home. In primary school, she gravitated towards math and science. Ally went on to receive her Bachelor of Science in Biological Engineering from Louisiana State University in 2013. She became interested in plant pathology during a summer internship at South Dakota State University when ash rust deviously infected her and her intern partner's cordgrass experiment subjects. Ally was offended and intrigued. At the behest of her internship advisor, she pursued the local introductory plant pathology course upon returning to Baton Rouge. She was delighted to find that Dr. Clayton Hollier and the Department of Plant Pathology & Crop Physiology were willing to accept her as a graduate student studying the sheath blight pathogen on rice.

Ally enjoys hands-on research, especially as a mixture of lab shenanigans and outdoor gallivanting. She plans to continue working to protect plants as a research associate under Dr. Stephen Harrison in the Louisiana State University School of Plant, Environmental & Soil Sciences (something about department names including ampersands). Ally's dream is to help feed the world with her future, collaborative, scientific efforts.